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Corresponding Author: Dr. István Pócsi,

Corresponding Author's Institution: University of Debrecen

First Author: Márton Miskei

Order of Authors: Márton Miskei; Zsolt Karányi; István Pócsi

Abstract: Stress-response proteins of Aspergillus nidulans, A. clavatus, A. flavus, A. fumigatus, A. niger, A. oryzae, A. terreus and Neosartorya fischeri (3908 in total) were annotated and grouped according to stress types (http://193.6.155.82/AspergillusStress/). All genomes harboured elements of the SskA - HogA/SakA stress signalling pathway. There are accumulating data pointing at the importance of SskA - HogA/SakA signalling in different types of stress-responses in the aspergilli and, in this regard, these filamentous fungi are closer to fission yeast than to budding yeast. The abundance of annotated stress sensing histidine kinases and transcriptional regulators in each Aspergillus species indicates that the applicability of yeast-based models to fully describe and explain the stress-responses of these fungi is limited. Most excitingly, putative orthologues of both S. cerevisiae Msn2p/Msn4p C2H2 zinc finger-type and S. pombe Atf1 bZip-type 'general stress' transcription factors were annotated in the aspergilli, foreshadowing complex and robust stress defence systems in these euascomycetes.

Annotation of stress-response proteins in the aspergilli

Márton Miskei[a,b,#], Zsolt Karányi[c,#] and István Pócsi[a,*]

[a] - Department of Microbial Biotechnology and Cell Biology, Faculty of Science and Technology, University of Debrecen, H-4010 Debrecen, Hungary

[b] - Department of Horticulture and Plant Biotechnology, Faculty of Agricultural Science, University of Debrecen, H-4010 Debrecen, Hungary

[c] - First Department of Medicine, Faculty of Medicine, University of Debrecen, H-4012 Debrecen, Hungary

* - Corresponding author:
Department of Microbial Biotechnology and Cell Biology, Faculty of Science and Technology, University of Debrecen, P.O.Box 63, H-4010 Debrecen, Hungary; Tel: 36-52-512900 ext. 62337, Fax: 36-52-512925, E-mail: istvanpocsi@yahoo.com

[#] - These two authors contributed equally to this paper.

Abstract

Stress-response proteins of *Aspergillus nidulans*, *A. clavatus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. oryzae*, *A. terreus* and *Neosartorya fischeri* (3908 in total) were annotated and grouped according to stress types (http://193.6.155.82/AspergillusStress/). All genomes harboured elements of the SskA - HogA/SakA stress signalling pathway. There are accumulating data pointing at the importance of SskA – HogA/SakA signalling in different types of stress-responses in the aspergilli and, in this regard, these filamentous fungi are closer to fission yeast than to budding yeast. The abundance of annotated stress sensing histidine kinases and transcriptional regulators in each *Aspergillus* species indicates that the applicability of yeast-based models to fully describe and explain the stress-responses of these fungi is limited. Most excitingly, putative orthologues of both *S. cerevisiae* Msn2p/Msn4p C2H2 zinc finger-type and *S. pombe* Atf1 bZip-type 'general stress' transcription factors were annotated in the aspergilli, foreshadowing complex and robust stress defence systems in these euascomycetes.

Introduction

Comparative genome analysis is flourishing in the aspergilli (Jones, 2007) with four genome sequences published (*Aspergillus nidulans*, a filamentous fungus model organism, Galagan et al., 2005; *Aspergillus fumigatus*, the most common species among the aspergilli causing human infections, Nierman et al., 2005; *Aspergillus oryzae*, a species traditionally used in the Japanese and Chinese cuisines to ferment food, Machida et al., 2005; *Aspergillus niger*, an industrial organic acid and enzyme producer, Pel et al., 2007) and four others assembled and annotated (*Aspergillus clavatus*, a patulin producer close relative of *A. fumigatus*, Wortman et al., 2006; *Aspergillus flavus*, an aflatoxin producer and opportunistic pathogen fungus, Payne et al., 2006; *Aspergillus terreus*, a source of the serum cholesterol lowering compound lovastatin, Wortman et al., 2006, *Neosartorya fischeri* (teleomorph of *Aspergillus fischerianus*), a soil-inhabiting close relative of *A. fumigatus*, Wortman et al., 2006). A most recent comparative genomic study on the opportunistic human pathogenic fungus *A. fumigatus* and two closely related, rarely pathogenic species *N. fischeri* and *A. clavatus* led to the identification of genomic islands in the pathogen, which function as designated gene dumps and gene factories (Fedorova et al., 2008). Other comparative genomics studies shed light on mating process genes in *A. oryzae* and *A. fumigatus* (Galagan et al., 2005; Nierman et al., 2005), the gliotoxin gene cluster in *A. fumigatus* (Gardiner and Howlett, 2005), and elements of programmed cell death pathways in the aspergilli (Fedorova et al., 2005).

Free-living fungi often encounter different kinds of environmental stress including changes in osmolarity, temperature, availability of O_2 and nutrients (Gasch, 2007). Pathogens are exposed to chronic oxidative stress caused by immune system cells (Fekete et al., 2007), and industrial fungi cope with stress coming from the technology employed, e.g. the use of elevated O_2 pressure (Kubicek and Karaffa, 2006). A deeper understanding of stress signalling and regulation in filamentous fungi may help us to find new targets for future antifungal drug design (Rementeria et al., 2005; Meyer et al., 2007), to develop more stress-tolerant and high producer industrial strains (Emri et al., 1997, 1999; MacKenzie et al., 2005; Pócsi et al., 2005), and to attenuate mycotoxin production (Pócsi et al., 2005; Kim et al., 2008).

Environmental stress response (ESR) genes responding to diverse types of stress have been identified in *Saccharomyces cerevisiae* (Gasch et al., 2000; Causton et al., 2001) and *Schizosaccharomyces pombe* (Chen et al., 2003) but a common stress response is less obvious in the opportunistic and dimorphic human pathogen *Candida albicans* (Enjalbert et al., 2003; Gasch, 2007). Meanwhile stress signalling is stress-specific in budding yeast, *e.g.* the Hog1p MAPK signalling pathway is responsive to osmotic and related stress, Sty1 "all-purpose" MAPK pathway plays a pivotal role in stress signalling in *S. pombe* (Gasch et al., 2000; Causton et al., 2001; Chen et al., 2003; Gasch, 2007). Stress signalling pathways seem to converge at Msn2p/Msn4p and Atf1 "general stress" transcriptional factors in budding and fission yeasts, respectively, regulating similar groups of ESR genes (Gasch et al., 2000; Causton et al., 2001; Chen et al., 2003; Gasch, 2007).

In this study, we aim at the identification of *Aspergillus* orthologues of yeast stress adaptation proteins screening the genomes of eight *Aspergillus* spp. (*A. nidulans*, *A. clavatus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. oryzae*, *A. terreus* and *N. fischeri*). Moreover, we intend to compare the groups of stress-response proteins annotated for each *Aspergillus* to find species-specific elements. Finally, we discuss the hypothesis that Hog1p MAPK signalling is not limited to osmotic stress in the aspergilli (Kawasaki et al., 2002; Xue et al., 2004; Du et al., 2006; Hagiwara et al., 2007a) and may play a more common role in the orchestration of different stress responses similar to that of *S. pombe* Sty1 (Gasch, 2007).

Materials and Methods

Setting up databases. Databases were created including all proteins (and translated genes) that may play any role in stress response in yeasts and filamentous fungi. In the first database (Database No. 1), stress-response proteins were extracted from *S. cerevisiae*, *S. pombe* and *C. albicans* AMIGO databases (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi). Following that Database No. 1 was supplemented with stress-response proteins of the filamentous fungi *Neurospora crassa*, *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger* and *A. oryzae*. Filamentous fungus stress-response proteins were identified by web-search in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) using the 'genus name+stress' and 'genus

name+DNA repair' key word pairs, and protein sequences were collected through the web-links found in the corresponding publications (http://www.ncbi.nlm.nih.gov/).

In the second database (Database No. 2), all putative proteins coming from *A. nidulans* translated ORFs were assessed using the Broad Institute *Aspergillus nidulans* database http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/Home.html. In Database No. 3, putative protein sequences available for *S. cerevisiae* (http://www.yeastgenome.org/), *S. pombe* (http://www.sanger.ac.uk/Projects/S_pombe/), *C. albicans* (http://www.candidagenome.org/), *N. crassa* (http://www.broad.mit.edu/annotation/genome/) and the aspergilli *A. flavus*, *A. fumigatus*, *A. niger* and *A. oryzae* (http://www.broad.mit.edu/annotation/genome/) were summarized (for genome sequencing and annotation of yeasts and filamentous fungi consult the papers of Cherry et al., 1997; Mewes et al., 1997; Ashburner et al., 2000; Wood et al., 2002; Galagan et al., 2003, 2005; Arnaud et al., 2005; Machida et al., 2005; Nierman et al., 2005; Aslett and Wood, 2006; Payne et al., 2006 and Pel et al., 2007).

Homology search and annotation of *A. nidulans* stress proteins. The following five-step protocol was employed:

- *A. nidulans* homologues of stress-response proteins were identified by BLASTP search program (Altschul et al., 1997; softwares used: blastall and formatdb downloaded from http://www.ncbi.nlm.nih.gov/Ftp) by comparing stress-response proteins from Database No. 1 to putative proteins in Database No. 2.
- Results were filtered according to the 1E-40 (stands for $1x10^{-40}$) expectation value (*E*) cut-off criteria (Ayoubi et al., 2002; Xu et al., 2003; Poustka et al., 2003; Pócsi et al., 2005). The *E*-value is a parameter widely used for quantifying sequence similarities and assessing underlying biological relationships (Joshi and Xu, 2007). In general, the lower the *E*-value, the more likely the homology and the biological relationship between the compared sequences (Joshi and Xu, 2007).
- To reduce the number of mis-annotated proteins, sequences of candidate *A. nidulans* stress-response proteins were compared to putative proteins in Database No. 3 using BLASTP protein sequences alignment.
- Results were filtered again at *E*-value 1E-40.

- When the highest homology protein sequence found in the fourth step was identical to the protein we started the homology search with the result was accepted and discussed. In any other case, the outcome of the homology search was disregarded.

At last, *A. nidulans* orthologues were collated, characterized and grouped using Gene Ontology terms (Gene Ontology Consortium, 2001; Dwight et al., 2002; http://www.geneontology.org/; http://www.yeastgenome.org/) and supplemented with literature data found in species-specific genomic databases and NCBI PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez).

Screening for stress-response proteins in the genus *Aspergillus*. All putative proteins translated from *Aspergillus clavatus, A. flavus, A. fumigatus, A. nidulans, A. niger, A. oryzae, Aspergillus terreus* and *Neosartorya fischeri* ORFs (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005; Payne et al., 2006; Wortman et al., 2006, Pel et al., 2007) were assessed at http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html to set up Database No. 4. Stress-response proteins from Database No. 1 were compared to putative proteins in Database No. 4 with the sequence search program BLASTP. After filtering rough data using the $E \leq$ 1E-40 cut-off criteria, candidate *Aspergillus* stress proteins and putative proteins in Database No. 3 were compared using BLASTP. Further annotation steps were the same as described above for *A. nidulans* stress-response proteins.

Results

A. nidulans special and *Aspergillus* generic stress databases are available at web links http://internal.med.unideb.hu/annota20070815/ (release date September 2007) and http://193.6.155.82/AspergillusStress/ (release date May 2008), respectively, and annotated *A. nidulans* stress-response proteins were also submitted to The J. Craig Venter Institute *Aspergillus nidulans* Annotation Database (http://www.tigr.org/; date of submission September 2007). *Aspergillus* stress-specific datasets are also linked directly to this paper (Supplementary Tables 1-8). In all stress databases and datasets presented in this paper, general information is given concerning annotated proteins (*Aspergillus* locus IDs) and their closest homologues in other species (stress gene locus IDs and protein names), stress type,

gene ontology (GO) classes (GO IDs and GO descriptions), relevant publications (PMID IDs) and a brief functional characterization. Only stress-related GO terms were used to describe the function of annotated gene products to facilitate future computer-based data mining. In the *Aspergillus* stress database (http://193.6.155.82/AspergillusStress/), clicks on locus IDs and PMID IDs lead to the appropriate Broad Institute database entry and PubMed reference page, respectively.

The total number of annotated stress-response proteins in *A. nidulans* at $E \leq$ 1E-40 comparison stringency is 486 (Supplementary Table 1), from which 133 takes part in osmotic stress, 108 in oxidative, 85 in thermal stress and unfolded protein, 37 in starvation stress responses, 172 in DNA repair processes and 73 in other stress responses (Table 1; Supplementary Table 1). Because only a very limited number of *A. nidulans* stress-response genes and gene products have been characterised satisfactorily thus far GO terms used for the description of orthologous yeast proteins are presented in Supplementary Table 1 even if the corresponding *A. nidulans* genes and/or proteins have already been published.

Considering other *Aspergillus* species, the numbers of putative stress response proteins were very similar to that found for *A. nidulans*, namely 484 for *A. clavatus*, 520 for *A. flavus*, 471 for *A. fumigatus*, 428 for *A. niger*; 503 for *A. terreus*, 515 for *A. oryzae* and 501 for *N. fischeri*. Altogether, 3908 *Aspergillus* stress-response proteins were annotated in this project. These data were also grouped according to functional categories and are summarized in Supplementary Tables 2-8.

A model of osmotic and oxidative stress response signalling and regulation in *A. nidulans* was set up collating annotation data presented in Supplementary Table 1 and relevant literature data published primarily for yeasts (Figure 1). Annotated elements of two-component His-to-Asp phosphorelay stress signalling systems, down-stream MAPK regulatory pathways and elements of osmotic and oxidative stress defences are summarized for all aspergilli studied in Table 2. Putative *A. nidulans* orthologues with relatively low homology (1E-09 > E > 1E-40) to yeast osmotic and oxidative stress-response proteins are presented in Table 3.

Discussion

Osmotic and oxidative stress response proteins

The great majority of putative stress response proteins of the aspergilli were annotated using yeast, primarily *S. cerevisiae* and *S. pombe*, data because our current knowledge on the stress response machinery of filamentous fungi is rather incomplete and mosaic-like in general. Not surprisingly, the organisation and regulation of stress sensing, signalling and response pathways in yeasts (Hohman 2002; O'Rourke et al., 2002; Chen et al., 2003; Saito and Tatebayashi 2004; Chauhan et al., 2006; Krantz et al., 2006a,b; Gasch et al., 2007) serve as a benchmark for the evaluation of stress response systems of other fungi. At first, we summarize the elements of osmotic and oxidative stress response in yeasts to make the presentation and discussion of *Aspergillus* stress protein annotation data easier and more effective.

High Osmolarity Glycerol (HOG) stress signalling and stress response in yeasts. Yeasts can sense and respond to high osmolarity stress *via* mitogen-activated protein kinase (MAPK) signalling systems (Table 2, Figure 1). In *S. cerevisiae*, Hog1p MAPK is activated by two independent upstream mechanisms. The first branch includes a two-component His-to-Asp phosphorelay system including Sln1p histidine sensor kinase, Ypd1p histidine-containing phosphotransfer protein and Ssk1p and Skn7p response regulators (Posas et al., 1996; Li et al., 1998). In this pathway, the phosphorylation of Ssk1p prevents any interaction with Ssk2p/Ssk22p MAPKKK keeping the downstream elements of the Ssk2p/Ssk22p - Pbs2p - Hog1p MAPK pathway inactive (Posas and Saito, 1998). Hyperosmotic stress inhibits Sln1p leading to the dephosphorylation of Ypd1p and Ssk1p, which facilitates Ssk1p - Ssk2p/Ssk22p interactions and the activation of Hog1p (Posas and Saito, 1998).

The second branch includes the Sho1p transmembrane sensor kinase, and the formation of Sho1p, Cdc42p, Ste20p/Cla4p, Ste50p, Ste11p (MAPKKK), Pbs2p (MAPKK) and Hog1p (MAPK) multi-component signalling complex (Saito and Tatebayashi 2004; Tatebayashi et al., 2006). The assembly of the complex includes the activation of Ste11p by Ste20p/Cla4p kinases by indirect docking *via* Ste50p (a SAM domain-containing protein) and Cdc42p (a small Rho-like GTPase), and the activation of Pbs2p by Ste11p by indirect docking *via*

Ste50p adaptor and Sho1p (Tatebayashi et al., 2006). According to a recent publication of Tatebayashi et al. (2007), Hkr1p and Msb2p mucin-like transmembrane proteins are the potential osmosensors for the Sho1p branch of Hog1p activation.

Hog1p normally localizes in the cytoplasm in unstressed cells but translocates to the nucleus after phosphorylation in cells exposed to osmotic stress. Nuclear import of Hog1p requires the activity of Gsp1p small GTP-binding protein and the importin β homologue Nmd5p (Ferrigno et al., 1998). Dephosphorylated Hog1p is exported from the nucleus to the cytoplasm by the nuclear exchange sequence receptor protein Crm1p (Ferrigno et al., 1998). Importantly, the Ypd1p phosphotransfer protein also needs to shuttle between the cytoplasm and the nucleus to phosphorylate the response regulators Ssk1p (cytoplasm) and Skn7p (nucleus) (Lu et al., 2003).

Dephosphorylation of Hog1p is important to avoid the deleterious effects of the hyperactivation of this MAPK (Saito and Tatebayashi 2004). Important negative regulators are Ptp2p and Ptp3p protein phosphotyrosine phosphatases dephosphorylating Hog1p in both the nucleus (Ptp2p) and the cytoplasm (Ptp3p) (Mattison and Ota, 2000) and the protein phosphatase type 2C enzymes Ptc1p, Ptc2p and Ptc3p (Warmka et al., 2001; Young et al., 2002). Ptc1p dephosphorylates Hog1p *via* a docking interaction between Ptc1p, Hog1p, Pbs2p and the small adaptor protein Nbp2p (Mapes and Ota, 2004; Saito and Tatebayashi, 2004).

There are several different Hog1p-dependent activation mechanisms of osmotic stress regulated genes, including (i) the phosphorylation of Sko1p bZip-type repressor, which leads to the disassembly of Tup1p-Cyc8p(Ssn6p)-Sko1p repressor complexes and the activation of Sko1p (Proft et al., 2001), (ii) the Hot1p-dependent activation of the RNA polymerase II complex by Hog1p (Alepuz et al., 2003) and (iii) the Msn2p/Msn4p 'general stress' C2H2 zinc finger transcription factors (Moskvina et al., 1998; Gasch et al., 2000, Causton et al., 2001; Gasch, 2007) dependent recruitment of Hog1p to osmotic stress responsive promoters (Reiser et al., 1999; Alepuz et al., 2001; Rep et al., 2000; O'Rourke et al., 2002). Importantly, the nuclear localization of Msn2p/Msn4p is independent of Hog1p and is regulated by protein kinase A (Görner et al., 1998). A group of stress-response genes, *e.g. CTT1* encoding cytosolic catalase T, is also regulated by the transcriptional activator Msn1p (Rep et al., 1999).

Other Hog1p targets include Sgd1p nuclear protein (Akhtar et al., 2000; Lin et al., 2002) and the Smp1p transcription factor (de Nadal et al., 2003). The Rck2p protein kinase is a substrate of Hog1p and participates in G_2 checkpoint control and the osmotic stress triggered attenuation of protein synthesis (Bilsland-Marchesan et al., 2000; Teige et al., 2001). In addition, Hog1p recruits Rpd3p histone deacetylase to induce gene expression on osmotic stress (de Nadal et al. 2004).

In *S. pombe*, a homologue of the Ssk2p/Ssk22p - Pbs2p - Hog1p MAPK kinase pathway does exist, which consists of Wak1/Win1 (MAPKKK) - Wis1 (MAPKK) - Sty1 (syn. Spc1) (MAPK) protein kinases (Figure 1). MAPK signalling is stimulated by a wide spectrum of environmental stress in addition to osmotic stress in this yeast (Shieh et al., 1997; Hohmann 2002; Chen et al., 2003; Gasch, 2007). The MAPK pathway is activated by a two-component His-to-Asp phosphorelay system including Mak1, Mak2 and Mak3 histidine kinases, Mpr1 phosphotransfer protein and Mcs4 response regulator, when cells are facing oxidative stress (Buck et al., 2001; Nakamichi et al., 2003). The sensor system for osmotic stress is not known yet (Hohmann, 2002) because fission yeast's genome does not accommodate either *SLN1* or *SHO1* orthologues (Krantz et al., 2006a). In fission yeast, the transcription factor Atf1 is a substrate of Sty1 (Shiozaki and Russel, 1996; Wilkinson et al., 1996), and phosphorylated Atf1 is a major key player in the induction of a wide spectrum of stress-responsive genes (ESR genes; Chen et al., 2003; Gasch, 2007; Sansó et al., 2008). Sty1 is dephosphorylated by Pyp1 and Pyp2 protein phosphotyrosine phosphatases (Millar et al., 1995) and, interestingly, Atf1 directly activates *pyp2* gene completing a negative feed-back loop to phosphorylated Sty1 (Figure 1; Wilkinson et al., 1996). It is noteworthy that fission yeast does not have any orthologue of budding yeast's Msn2p/Msn4p transcription factors (Gasch, 2007).

Response regulators in addition to Ssk1p (*S. cerevisiae*) and Mcs4 (*S. pombe*) are Skn7p and Prr1 transcription factors, respectively, which are phosphorylated by Ypd1p (*S. cerevisiae*) and Mpr1 (*S. pombe*) phosphotransfer proteins. Skn7p is phosphorylated in the nucleus by Ypd1p (Lu et al., 2003). Skn7p and Prr1 govern the expression of an array of stress-responsive genes mainly required in oxidative stress defence (Krems et al., 1996; Ohmiya et al., 1999). It is noteworthy that Skn7p response regulator and Yap1p bZip transcription factor co-operate in the induction of numerous oxidative stress responsive genes in budding yeast (Morgan et al., 1997).

There are numerous stress-responsive genes under Hog1p (Sty1) control in yeasts including glycerol biosynthetic genes like *GPD1* (glycerol-3-phosphate dehydrogenase; Ansell et al., 1997) and *GPP2* (glycerol-3-phosphatase; Boy-Marcotte et al. 1998), the glycerol transporter gene *STL1* (de Nadal et al., 2003), *ALD2* and *CTT1* encoding cytoplasmic aldehyde dehydrogenase and cytosolic catalase T, respectively (Márquez et al., 1998), as well as P-type ATPase sodium pump genes like *ENA1* and *ENA2* (Garciadeblas et al., 1993) and *pmp3* coding for a small membrane protein (Wang and Shiozaki, 2006).

Control by Skn7p (Prr1) response regulator in osmotic stress defence was demonstrated at genes *cta3* (intracellular cation transporter; Greenall et al., 2002) and *OCH1* (mannosyltransferase of the cis-Golgi apparatus; Li et al., 2002) but its main function, together with Yap1p, is the regulation of oxidative stress defence genes like *GPX2* (glutathione peroxidase; Tsuzi et al., 2004), *CTT1* (cytosolic catalase T; He and Fassler, 2005), *TRX2* and *TRR1* (thioredoxin and thioredoxin reductase; Lee et al., 1999). In fission yeast, the expression of antioxidative genes like *gpx1* and *ctt1* is Atf1 and/or Pap1 (a Yap1p orthologue) transcription factor-dependent (Yamada et al., 1999; Nakagawa et al., 2000).

In the opportunistic human pathogenic dimorphic fungus, *C. albicans*, a functioning Ssk2p-Pbs2p-Hog1p MAPK signalling pathway was found, which transmits both osmotic and oxidative stress signals (Chauhan et al., 2003, 2006; Arana et al., 2005; Monge et al., 2006; Cheetham et al., 2007; Walia and Calderone, 2008). Meanwhile oxidative stress signals may be generated in two-component phosphorelay system consisting of a histidine kinase (Sln1p, Nik1p, Chk1p) and the response regulator Ssk1p (Li et al., 2004; Roman et al., 2005; Cheetham et al., 2007) the molecular background of osmotic stress signalling has remained yet to be elucidated (Cheetham et al., 2007). Sho1p-Ste11p signalling regulates cell wall biogenesis and morphogenesis via Cek1p MAPK pathway in this fungus (Román et al., 2005; Monge et al., 2006). Ssk2p-Pbs2p-Hog1p-type MAPK pathway is also present in another human pathogen, *Cryptococcus neoformans* (Bahn et al., 2007).

Osmotic and oxidative stress signalling in *A. nidulans* - similarity and differences with yeast stress signalling and response systems. Similar to the stress-responsive Sty1 - Hog1p MAPK pathways of *S. pombe* and *C. albicans*, the SskB - PbsB - HogA (syn. SakA) MAPK pathway of *A. nidulans* (Figure 1, Table 2) transmits osmotic (Han and Prade, 2002; Kawasaki et al., 2002; Furukawa et al., 2005; Hagiwara et al., 2007a; Vargas-Pérez et al.,

2007) and oxidative (Kawasaki et al., 2002; Furukawa et al., 2005; Hagiwara et al., 2007a) stress signals and is activated by the response regulator SskA (Hagiwara et al., 2007a). It is worth noting that the *ΔsskA* mutants of Vargas-Pérez et al. (2007) resisted H_2O_2 up to 2 mM and were capable of growing even in the presence of 4 mM H_2O_2 but reduced induction of a *catB*:*lacZ* reporter gene fusion in *ΔsakA* background was observed by the same authors. On the contrary, the *ΔsskA* mutant of Hagiwara et al. (2007a) possessed a hypersensitive phenotype to oxidative stress particularly to that caused by H_2O_2. These observations together with the transient phosphorylation of SakA under both osmotic and oxidative stress strengthen the view that the SskA - SakA (syn. HogA) system transduces different types of stress signals (Kawasaki et al., 2002; Furukawa et al., 2005). Importantly, *sakA* complemented *Δspc1* (syn. *sty1*) defects in *S. pombe* and the gene product was phosphorylated in response to both osmotic and oxidative stress in fission yeast (Kawasaki et al., 2002).

Other likely upstream components of stress signalling *via* SskA-dependent His-to-Asp phoshorelay system are YpdA phosphotransfer protein (Furukawa et al., 2005; Vargas-Perez et al., 2007) and NikA, a Mak2-type histidine kinase, which transmits fungicide signals but does not seem to be indispensable in either oxidative or osmotic stress responses (Hagiwara et al., 2007b; Vargas-Pérez et al., 2007) (Figure 1, Table 2). Interestingly, the homology between *S. cerevisiae* Ypd1p and *A. nidulans* YpdA is moderate with an *E*-value of 5E-18 (Table 3) but *ypdA* complemented *ΔYPD1* mutation in *S. cerevisiae* (Furukawa et al., 2005).

Another candidate to trigger osmotic stress signals in *A. nidulans* is the TcsB histidine kinase, an orthologue of yeast Sln1p (Catlett et al., 2003; Table 2), which complements *Δsln1* defect in *S. cerevisiae* (Furukawa et al., 2002). Although the *ΔtcsB* gene deletion mutant did not exhibit any osmosensitive phenotype (Furukawa et al., 2002) the *A. nidulans* TcsB - YpdA - SskA two-component system may possess a physiological function similar to that of the yeast Sln1p-Ypd1p-Ssk1p proteins (Furukawa et al., 2005). Unlike in yeasts and similar to filamentous fungus pathogens (Catlett et al., 2003), the *A. nidulans* genome contains as many as 15 genes coding for histidine kinases (Vargas-Pérez et al., 2007; Suzuki et al., 2008), and this considerable redundancy may explain the absence of any clear-cut phenotype in the *ΔtcsB* mutant. Interestingly, another non-essential sensor kinase, TcsA, may be required to produce conidia under different kinds of oxidative stress (Appleyard et al., 2000) but TcsA - YpdA - response regulator signalling pathway has not been demonstrated. Other *A. nidulans* histidine

kinases with considerable homologies to *S. pombe* Mak1 and Mak2 are the putative gene products of AN3101.3 and AN3102.3 ORFs, respectively (Table 2).

The *A. nidulans* SskB - PbsB - HogA/SakA MAPK pathway is activated only by two-component signalling because PbsB lacks a typical Pro-rich motif, which is required for binding Pbs2p to Sho1p in budding yeast (Zarrinpar et al., 2003; Furukawa et al., 2005). Considering that the Pro-rich Sho1p-binding domain is also absent in *C. albicans* Pbs2p MAPKK it is reasonable to assume that the primary role of Sho1p-signalling is not in osmosensing but in morphogenesis (Krantz et al., 2006a). In good accordance with this, other *A. nidulans* proteins with similarity to elements of the yeast Sho1p branch play important roles in hyphal morphogenesis (ModA, a Cdc42p-like protein; Virag et al., 2007) or sexual development (SteC, a Ste11p-like protein; Wei et al., 2003). It is noteworthy that the homologies between yeast Sho1p, Msb2p, Ste50p (important elements of Sho1p-dependnet stress signalling) and their counterparts in *A. nidulans* were characterized with *E*-values around or above the $E \leq$ 1E-40 cut-off (6E-17 - 9E-37, Table 3, and 1E-32 - 8E-42 for putative Sho1p orthologues in the aspergilli).

Another spectacular difference between Hog1p-dependent and HogA/SakA-dependent stress response regulatory pathways is that PbsB MAPKK activates another Hog1p orthologue, MpkC, in *A. nidulans*, which is absent in yeasts, and the overexpression of which suppresses the high-osmolarity sensitivity of *ΔhogA* (Furukawa et al., 2005). On the other hand, MpkC is dispensable in osmoadaptation (Furukawa et al., 2005).

An orthologue of Prr1 (*S. pombe*) and Skn7p (*S. cerevisiae*) response regulators, SrrA, also functions in *A. nidulans* (Hagiwara et al., 2007a; Vargas-Pérez et al., 2007) (Figure 1, Table 2). SrrA is required for oxidative stress (especially against H_2O_2 and *t*BOOH) and, slightly, for osmotic stress resistances (Hagiwara et al., 2007a; Vargas-Pérez et al., 2007).

No information is available at the moment on the nuclear import and export of phosphorylated HogA/SakA MAPK and YpdA histidine-containing phosphotransmitter although putative orthologues of Gsp1p-Nmd5p importin and Crm1p export factor can be found (Table 2). Moreover, an exportin candidate, KapK, has been shown to take part in the nuclear export of NirA nitrate assimilation transcription factor (Bernreiter et al., 2007). Similarly, little is known about the dephosphorylation of HogA/SakA but osmotic stress dependent up-

regulation of the putative protein phosphatase gene *ptpA* has been reported (Han and Prade, 2002).

Among the potential substrates of HogA/SakA, RpdA and AtfA (*E*-value for homology between *S. pombe* Atf1 and *A. nidulans* AtfA is 1E-36, Table 3) have been reported or hypothesized (Graessle et al., 2000; Aguirre et al., 2005). The *A. nidulans* RcoA shares sequence similarity with *S. cerevisiae* Tup1 and *N. crassa* RCO1 (Hicks et al., 2001), and it is another possible interacting partner of HogA/SakA. RcoA has pleitropic effects on vegetative growth, sexual and asexual spore production and sterigmatocystin biosynthesis but does not regulate carbon catabolite repression in *A. nidulans* (Hicks et al., 2001; Todd et al., 2006). One explanation for the reduced metabolic regulatory activity of RcoA may be the absence of Cyc8p(Ssn6p) and Sko1p co-repressor orthologues in the *A. nidulans* genome (Tables 1 and 2), which prevents the formation of repressor complexes similar to Tup1p-Cyc8p(Ssn6p)-Sko1p in budding yeast (Proft et al., 2001). Interestingly, other aspergilli harbour Cyc8p orthologues in their genomes (Table 2).

In budding yeast, Sko1p is the orthologue of *S. pombe*'s Atf1 'general stress' transcription factor (Gasch, 2007) but the activation of Sko1p bZip protein is only dependent on osmotic stress in *S. cerevisiae*, which role is quite minute in comparison to the cardinal role Msn2p/Msn4p C2H2 zinc finger proteins play in the regulation of ESR (Gasch et al., 2000; Gasch, 2007). The closest homologues of Sko1p are also AtfAs in the genomes of the aspergilli (Table 3) but these homologies are low. Therefore, further research is needed to answer the important question whether or not AtfAs are orthologues of Sko1p and will take over only the osmotic stress regulatory functions clearly attributed to Sko1p in *S. cerevisiae*. Or, alternatively, AtfAs are orthologues of fission yeast's Atf1 and occupy a central position in the coordination of ESR including oxidative stress (Aguirre et al., 2005).

Importantly, a Msn2p/Msn4p-type protein, MsnA (*E*-values for homology with budding yeast's Msn2p and Msn4p are 1E-16 and 2E-17, respectively; Table 3), was also annotated in *A. nidulans*, which was induced by versatile types of stress (Han and Prade, 2002). This opens the possibility of a complex co-regulation of stress response genes by Atf1-type bZip and Msn2p/Msn4p-type C2H2 zinc finger transcriptional factors in *A. nidulans*. A putative orthologue of *A. nidulans* MsnA has been characterized in *Trichoderma atroviride*, which is called Seb1 ('stress response element binding protein'; *E*-value for homology between *A.*

nidulans MsnA and *T. atroviride* Seb1 is 5E-55) and is involved in but not essential for osmotic stress defence (Peterbauer et al., 2002; Seidl et al., 2004). The expression of *T. atroviride seb1* did not respond to cadmium, pH and membrane perturbance stress, and overexpressed *seb1* cDNA did not complement a *S. cerevisiae MSN2*/*MSN4* deletion mutant (Peterbauer et al., 2002).

A series of stress-responsive genes normally subjected to Hog1p-Sko1p-dependent regulation under osmotic stress is controlled by the Yap1p transcription factor (a Hog1p-independent regulator) when budding yeast is exposed to oxidative stress (Gasch et al., 2000; Rep et al., 2001). By analogy, the bZip-type products of *A. nidulans* AN7513.3, called NapA (Asano et al., 2007), and *A. fumigatus* Afu6g09930, called AfYap1 (Lessing et al., 2007), also govern the expression of an array of oxidative stress responsive genes but the *E*-values between yeast Yap1p and the *A. nidulans* or *A. fumigatus* NapA and AfYap1 are 5E-13 and 4E-13, respectively, indicating relatively low homologies (Table 3). On the other hand, high-homology NapA (AfYap1) orthologues (expectation values 0.0) can be identified in all *Aspergillus* genomes screened.

Intriguingly, no Hot1p transcription factor (Alepuz et al., 2003) orthologue was found in the genomes of *A. nidulans* and other aspergilli (Table 3, Supplementary Tables 1-8). The lack of a Hot1p orthologue in *A. nidulans* may explain that *gfdA*, which is homologous to the yeast Hot1p-target Gpd1p (Rep et al., 2000), is not responsive to osmotic and oxidative stress (Han and Prade, 2002). The aspergilli's genomes do not accommodate any orthologues of Msn1p stress-response transcriptional activator (Rep et al., 1999) either (Table 3). Some other fungi responding to stress without the involvement of any Hot1p and Msn1p orthologues have been reported by Krantz et al. (2006a) and include *S. pombe*, *Yarrowia lipolytica, N. crassa* and *Ustilago maydis*.

The list of the potential target genes of HogA/SakA and/or SrrA-dependent regulations in *A. nidulans* includes the *GPD2* (*S. cerevisiae*) orthologue *gfdB* (Han and Prade, 2002; Furukawa et al., 2007), *enaA* (Han and Prade, 2002), *catB* (Hagiwara et al., 2007a; Vargas-Pérez et al., 2007) as well as *thiO*/*trxA* and *trxB*/*trxR* (Asano et al., 2007; Thön et al., 2007) (Table 2).

SskA response regulator is also implicated in stress-tolerant conidia formation because many trehalose and glycerol metabolic genes (*gfdA* and *gfdB*, glycerol-3-phosphate dehydrogenases;

gldB, glycerol dehydrogenase; *tpsA*, trehalose-6-phosphate synthase; *orlA*, trehalose-6-phosphate phosphatase; *treB*, neutral trehalase; Borgia et al., 1996; d'Enfert et al., 1999; Fillinger et al., 2001a, 2001b, Han and Prade, 2002; de Vries et al., 2003) are down-regulated in conidia of the *ΔsskA* mutant in addition to *catA* general stress tolerance catalase gene (Figure 1; Hagiwara et al., 2007a; Vargas-Pérez et al., 2007).

Osmotic and oxidative stress response in other aspergilli. Stress signalling and regulatory pathways are very similar in all aspergilli studied (Table 2; Supplementary Tables 1-8). *A. fumigatus* SakA MAPK plays a pivotal role in oxidative stress response of this opportunistic human pathogen meanwhile TcsB histidine kinase is dispensable (Du et al., 2006). MpkC MAPK signalling is important in carbon source utilization but not in high-osmolarity medium (Reyes et al., 2006). Considering Prr1/Skn7p-type response regulators, the *SKN7* orthologue of *A. fumigatus*, *afskn7*, was identified and *Δafskn7* showed a growth inhibition phenotype in the presence of H_2O_2 and *tert*-butylhydroperoxide (Lamarre et al., 2007). According to Sakamoto et al. (2008), the oxidative stress resistance gene *catA* is under AtfB bZip transcription factor (locus ID: AO090120000418) control in *A. oryzae* conidia, and *A. oryzae* genome also harbours an AtfA orthologue (Tables 2 and 3). Interestingly, *A. nidulans* and *A. terreus* do not have any AtfB orthologue obeying the E ≤ 1E-40 cut-off criteria but putative AtfB proteins encoded by the loci *A. nidulans* AN8643.3 and *A. terreus* ATEG_01978 were found with *E*-values 2E-20 and 9E-25, respectively. In other aspergilli, high-homology (E value < 1E-40) AtfB orthologues were annotated, encoded by the loci *A. flavus* AFL2G_08419 (3E-162), *N. fischeri* NFIA_074120 (2E-71), *A. fumigatus* Afu5g12960 (4E-77), *A. clavatus* ACLA_015960 (9E-79) and *A. niger* fge1_pg_C_8000498 (9E-56).

It is intriguing that there are two orthologues of the yeast Ssk1p (*A. nidulans* orthologue SskA) response regulator in the genome of *A. flavus* (ORFs AFL2G_06337 and AFL2G_12585, Table 2; *E*-values 4E-45 and 5E-20, respectively), and Rpd3p (*A. nidulans* orthologue RpdA) histone deacetylase also has two orthologues in this fungus (ORFs AFL2G_08263 and AFL2G_03062; *E*-values 2E-174 and 4E-139, respectively). Hence, this important aflatoxin-producer and opportunistic human pathogen seems to possess a more complex osmotic and oxidative stress defence system than other aspergilli.

The important citric acid, gluconic acid and hydrolyse producer fungus *A. niger* lacks the Sln1p-type (TcsB-type) histidine kinase heavily supporting the view that TcsB is dispensable in the aspergilli (Furukawa et al., 2002; Du et al., 2006). The lack of transmembrane-spanning histidine kinase is not unprecedented among fungi; the smut fungus *U. maydis* does not possess any apparent Sln1p orthologue similar to fission yeast (Krantz et al., 2006a,b).

Other types of stress

Thermal stress and Unfolded Protein Response (UPR). Although Hog1p/Sty1-type MAPKs are activated by heat stress as well in *S. pombe* (Degols et al., 1996; Samejima et al., 1997) and *N. crassa* (Noguchi et al., 2007) *A. nidulans* HogA/SakA does not seem to respond to thermal stress in vegetative tissue (Han and Prade, 2002; Kawasaki et al., 2002) but it is required for general stress tolerance, including heat stress, of germinated conidia (Kawasaki et al., 2002).

As far as the response regulator Skn7p is concerned, it interacts with the heat shock factor Hsf1p in *S. cerevisiae* to activate heat shock proteins under oxidative stress (Raitt et al., 2000). In the aspergilli, no orthologue of Hsf1p obeying the $E \leq$ 1E-40 cut-off rule was identified although putative HsfA proteins with 3E-23 - 2E-26 *E*-values were found (ORF IDs: *A. nidulans*: AN8035; *A. clavatus*: ACLA_003360; *A. flavus*: AFL2G_01739; *A. fumigatus*: Afu5g01900; *A. niger*: est_fge1_pg_C_7042; *A. oryzae*: AO090003001329; *A. terreus*: ATEG_09811; *N. fischeri*: NFIA_040210). Furthermore, no heat sensitive phenotype of *Δafskn7* was described in *A. fumigatus* (Lamarre et al., 2007).

In budding yeast, a large group of oxidative stress and heat shock responsive genes are regulated by Msn2p/Msn4p ‘general stress’ transcriptional factors (Gasch et al., 2000, Causton et al., 2001), and the expression of *A. nidulans msnA* was also reported to respond to heat by Han and Prade (2002). The participation of MsnA in the regulation of heat response genes is therefore foreseeable but needs verification.

Elements of RAS/Cyr1p (adenylate cyclase)/cAMP-dependent protein kinase A (RAS/cAMP/PKA) signalling and regulatory pathway (Estruch, 2000) have been annotated in the genomes of all aspergilli studied (Supplementary Tables 1-8) including orthologues of yeast Ras1p (Estruch, 2000), *N. crassa* CR-1 adenylate cyclase (Cruz et al., 1988) and yeast

Bcy1p cAMP-dependent protein kinase A regulatory subunit (Toda et al., 1987). RAS/cAMP/PKA signalling influences negatively the induction of Msn2p/Msn4p regulon by thermal stress and, hence, its reduced activity correlates well with developing thermotolerance in stress-exposed yeast cells (Görner et al., 1998; Garreau et al., 2000; Longo 2003). Both the catalytic (PkaC) and regulatory (PkaR) subunits of PKA of *A. niger* has been cloned and characterized (Benčina et al., 1997; Saudohar et al., 2002), and heat shock decreased markedly *pkaC* mRNA levels in this fungus (Benčina and Legiša, 2000).

As summarized in Supplementary Tables 1-8, aspergilli genomes also harbour putative orthologues of known cell integrity MAPKs (Mkc1p, *C. albicans*; Navarro-Garcia et al., 1995; Slt2p, *S. cerevisiae*; Hahn and Thiele, 2002), type 1 protein phosphatase (PP1C; Glc7p, *S. cerevisiae*; Tung et al., 1995), type 2C protein phosphatase (PP2C; Ptc1, Ptc2; *S. pombe*; Shiozaki and Russel, 1995), nucleoside diphosphate kinase (NDK-1; *N. crassa*; Yoshida et al., 2006) and calcineurin (CnaA; *A. oryzae*; Juvvadi et al., 2003) with predictably important roles in the heat shock response of this genus.

Targets of heat shock response regulators may include trehalose metabolic enzymes (TreB, *A. nidulans*, d'Enfert et al., 1999; TpsA and TpsB, *A. niger*, Wolschek and Kubicek, 1997; TpsA, *A. nidulans*, Fillinger et al., 2001) and catalases (CatA, *A. nidulans*, Noventa-Jordão et al., 1999; CatB, *A. nidulans*, Kawasaki et al., 1997; CatB, *A. oryzae*, Hisada et al., 2008). As has been shown most recently, the expression of conidia-specific *catA* is subjected to AtfB-dependent regulation in *A. oryzae* (Sakamoto et al., 2008).

Heat shock proteins play versatile roles to prevent the accumulation of denatured and aggregated proteins in stress-exposed cells (Riezman, 2004). In the aspergilli, putative orthologues of *N. crassa* HSP60 (Ostermann et al., 1989), HSP70 (Kapoor et al., 1995), HSP80 (Roychowdhury et al., 1992), *C. albicans* Hsp70p (La Valle et al., 1995), Hsp90p (Swoboda et al., 1995) and *S. cerevisiae* Hsp104p (Seppä et al., 2004) were annotated (Supplementary Tables 1-8). Misfolded mitochondrial proteins are likely degraded by the orthologue of the Pim1p protease (Wagner et al., 1994), and the removal of misfolded and denatured proteins is ubiquitinylation-dependent in the aspergilli under different stress conditions (Noventa-Jordão et al., 2000).

Among the annotated Unfolded Protein Response (UPR) proteins, all *Aspergillus* genomes contained orthologues of the yeast Ire1p and Kar2p proteins obeying the E ≤ 1E-40 cut-off criteria (Supplementary Tables 1-8). Any impairment of secretory and membrane protein folding in the ER, caused *e.g.* by heat shock, disulphide bond reducing agents, inhibitors of protein glycosylation, triggers UPR, which is initiated by the Ire1p (ER-stress sensor) - BiP (Kar2p; a Hsp70p-type Ire1p-modulator) system (Bertolotti et al., 2000; Kimata et al., 2007). The transmembrane protein Ire1p is a bifunctional enzyme (Ser/Thr kinase - endoribonuclease), the lumenal domain of which interacts with Kar2p molecular chaperone. Upon ER-stress, Kar2p binds unfolded protein and leaves Ire1p (Okamura et al., 2000), which undergoes aggregation and directly associates with unfolded proteins leading to its activation (Credle et al., 2005; Kimata et al., 2007). Activated Ire1p catalyses the splicing of *HAC1* premRNA allowing the synthesis of Hac1p, a bZip-type transcription factor, which is a major regulator of UPR in budding yeast (Mori et al., 1996; Sidrauski and Walter, 1997; Kimata et al., 2006). Putative Hac1p orthologues were also identified in all *Aspergillus* genomes studied but the homology between yeast and *Aspergillus* proteins was quite low (*E*-value 1E-12 - 6E-13).

Other annotated UPR proteins in the aspergilli are homologues of Lhs1p (*S. cerevisiae*; Mori et al., 1998) and calnexin (*S. pombe*; Cnx1; Parlati et al., 1995) molecular chaperons and Scj1p, a homologue of bacterial DnaJ, cooperating with Kar2p in yeast (Silberstein et al., 1998). The Ire1p sensor autophosphorylates itself under UPR (Kimata et al., 2007) and is dephosphorylated by Dcr2p phosphatase antagonizing UPR signalling (Guo and Polymenis, 2006). *A. nidulans* cyclophilin B (CypB) may also be an important factor in proper protein folding in ER under thermal stress (Joseph et al., 1999). Considering other subcellular structures, protein CgrA contributes to the protection of nucleolar integrity under heat stress in *A. fumigatus* (Bhabhra et al., 2006).

Enzyme production by recombinant aspergilli is of primary industrial importance considering the production of both native and heterologous proteins (Archer 1994; Davies 1994; Gouka et al., 1997; Archer and Peberdy, 1997; Guillemette et al., 2007). Because accumulation of misfolded heterologous proteins in the ER initiates UPR and, therefore, slows down protein secretion (‘secretion stress’) several elements and regulation of UPR are well-characterized even functionally in the aspergilli (Guillemette et al., 2007). For example, HacA transcription factors (Hac1p orthologues) have been cloned and characterized in *A. niger* (Al-Sheikh et al.,

2004; Mulder et al., 2004, 2006; Davé et al., 2006; Guillemette et al., 2007), *A. awamori* (Valkonen et al., 2003) and *A. oryzae* (Nakajima et al., 2006). Other described UPR elements in the aspergilli are BipA chaperones, (Kar2p orthologues; *A. niger*: van Gemeren et al., 1997; Punt et al., 1998; Al-Sheikh et al., 2004; Davé et al., 2006; *A. awamori*: van Gemeren et al., 1997, 1998; Punt et al., 1998; Lombraña et al., 2004; *A. oryzae*: Kasuya et al., 1999), protein disulphide isomerases (PdiA, *A. niger*: Ngiam et al., 2000; Al-Sheikh et al., 2004; PrpA, *A. awamori*: Wang and Ward, 2000; PdiA, *A. awamori*: Moralejo et al., 2001) and calnexin chaperone (ClxA, *A. niger*: Conesa et al., 2002).

Starvation. Nitrogen starvation causes G1-arrest in mitosis in fission yeast (Kumada et al., 1995) and triggers sexual differentiation (Nadin-Davis and Nasim, 1990; Sato et al., 1994; Peng et al., 2003). Several homologues of elements of fission yeast's sexual differentiation signalling and regulation can be found in the aspergilli as summarized in Supplementary Tables 1-8. Nutrient starvation also influences the asexual sporulation of *A. nidulans via* induction of BrlA, a sporulation-specific transcriptional regulator (Skromne et al., 1995). Regulatory elements of nutrient deprivation stress response may include HogA/SakA MAPK (Xue et al., 2004), putative orthologues of *N. crassa* RCO1 (Lee and Ebbole, 1998; orthologues in the aspergilli are RcoA proteins), *S. cerevisiae* Snf1p protein kinase (Kuchin et al., 2002) and Dpl1p sphingosine-1-phosphate lyase (Gottlieb et al., 1999). Target genes include homologues of yeast *MEP2* ammonium permease (Biswas and Morschhäuser, 2005), *ASP3* asparaginase (Bon et al., 1997), *YCSB* (*PRB1*; Teichert et al., 1989) and *isp6* (Nakashima et al., 2006) vacuolar proteases, *GGT1* γ-glutamyltranspeptidase I (Kim et al., 2005), and *A. nidulans* alcohol dehydrogenase II (ADHII; Jones et al., 2001). Phosphor-starvation seems to induce nucleotide phosphatases like Npp1p (Kennedy et al., 2005).

Other stress. Genes encoding glutathione S-transferases (Fraser et al., 2002; Burns et al., 2005) are present in the genomes of the aspergilli, and *A. fumigatus* GstA, GstB and GstC also possess glutathione peroxidase activity (Burns et al., 2005). Glutathione S-transferases play pivotal roles in oxidative stress defence and the detoxification of xenobiotics and heavy metals (Fraser et al., 2002; Pócsi et al., 2004; Burns et al., 2005).

The aspergilli also have orthologues of the yeast flavohemoglobin Yhb1p (Supplementary Tables 1-8), which is the centrepiece of nitrosative stress defence in both *S. cerevisiae* (Liu et

al., 2000; Horan et al., 2006) and the opportunistic pathogen *C. albicans* (Ullmann et al., 2004; Hromatka et al., 2005).

DNA repair. Elements of DNA repair systems were more recently annotated and discussed in details in *A. nidulans* by Goldman and Kafer (2004). Although a detailed description of DNA repair systems in aspergilli was beyond the scope of this paper gene annotations were completed and DNA repair data are summarized in Supplementary Tables 1-8. Transcriptome analyses of *A. nidulans* exposed to camptothecin (a topoisomerase I inhibitor) and *A. nidulans atmA* (an orthologue of human *ATM*, encoding a phosphatidyl-3-kinase-related protein kinase, a central regulator of DNA damage response) null mutant are also available in the literature demonstrating the complexity of DNA damage responses and the multiple pathways mediating DNA repair in the aspergilli (Malavazi et al., 2006, 2007).

ESR in the aspergilli. Unfortunately, whole-genome analyses of general stress responses have remained yet to be performed in the aspergilli (Gasch, 2007). Although many elements of stress sensing, signalling and response typical of yeasts are present in the *Aspergillus* genomes and, hence, ESR-like responses are foreseeable, still little is known about the organisation and hierarchy of these elements. As a result of previous observations and this annotation study, proteins of SskA - HogA/SakA stress signalling and regulation pathways are present and may be cardinal in the regulation of different types of stress responses (*e.g.* responses to osmotic, oxidative, starvation and even heat stress in germinating conidia) in all aspergilli and, in this regard, these euascomycetes are closer to the fission yeast *S. pombe* than to the budding yeast *S. cerevisiae* (Gasch, 2007). This view is further strengthened by the observation that the *S. cerevisiae* Sho1p-type stress signalling does not function in *A. nidulans*, and this transmembrane sensor kinase in the upstream branch of Hog1p MAPK pathway is absent in fission yeast. This means that Sty1/HogA MAPK pathway is activated only by two-component signalling in both fission yeast and the aspergilli.

The abundance of annotated histidine kinases, MAPKs (HogA/SakA, MpkC), response regulators (two SskAs in *A. flavus*) and transcriptional regulators, *e.g.* AtfA, AtfB, NapA (AfYap1), MsnA (no orthologue in fission yeast!), RpdA (two orthologues in *A. flavus*), may be indicative of a complex and robust stress defence system controlled by a high-complexity regulatory network in these filamentous fungi. The absence of orthologues of important transcriptional regulators like Sko1p, Hot1p and Msn1p in the aspergilli and Cyc8p in *A.*

nidulans together with the relatively low (*E* > 1E-40) homologies between yeast and *Aspergillus* Ypd1p – YpdA, Msn2p/Msn4p – MsnA, Atf1 – AtfA, Yap1p – NapA (AfYap1), Hsf1p – HsfA and Hac1p – HacA proteins challenges the view that yeast stress response systems can easily be reconstructed *in silico* in the aspergilli. Hence, these findings shed light on the limits of yeast-based models in filamentous fungus genome annotations.

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Tables

Table 1. Stress-response proteins annotated in different aspergilli

Species	Osmotic stress	Oxidative stress	Thermal stress and unfolded protein response	Starvation	DNA repair	Other stress	Stress-response proteins[a]
A. nidulans	133	108	85	37	172	73	**486**
A. clavatus	130	102	85	38	174	77	**484**
A. flavus	143	126	94	46	172	77	**520**
A. fumigatus	126	102	84	40	169	72	**471**
A. niger	117	113	73	32	142	50	**428**
A. oryzae	146	123	90	47	167	74	**515**
A. terreus	134	123	83	41	172	76	**503**
N. fischeri	134	124	88	38	179	73	**501**
***Aspergillus* species altogether[a]**	**1063**	**921**	**682**	**319**	**1347**	**572**	**3908**

[a] - One protein may be placed in several groups of stress response proteins and, hence, the annotated 3908 proteins appeared 4904 times in the six stress protein groups (Supplementary Tables 1-8).

Table 2.
Annotated elements and targets of SskA and SrrA response regulator-dependent osmotic and oxidative stress response regulatory pathways in the aspergilli.

Physiological function	Yeast stress-response proteins[a]	Orthologues and putative orthologues in the aspergilli							
		A. nidulans	*A. clavatus*	*A. flavus*	*A. fumigatus*	*A. niger*	*A. oryzae*	*A. terreus*	*N. fischeri*
Sho1p-like stress sensing and signalling branch[b]	Sho1p	AN7698 (ShoA)	ACLA_012130	AFL2G_06338	Afu5g08420	e_gw1_14.461	AO090701000763	ATEG_08258	NFIA_008170
	Cdc42p	AN7487.3 (ModA)	ACLA_068600	AFL2G_09325	Afu2g05740	An02g14200	AO090001000693	ATEG_06763	NFIA_082460
	Cla4p	AN8836.3	ACLA_009160	-	Afu5g05900	est_GWPlus_C_160600	AO090009000674	ATEG_09444	NFIA_037230
	Ste20p	AN2067.3	ACLA_089970	AFL2G_02712	Afu2g04680	est_GWPlus_C_40790	AO090003000267	ATEG_06035	NFIA_081350
	Ste11p	AN2269.3 (SteC)	ACLA_009750	AFL2G_10116	Afu5g06420	An17g01280	AO090009000610	ATEG_09389	NFIA_036710
Histidine kinase	Sln1p	AN1800.3 (TcsB)	ACLA 094100	AFL2G 04890	Afu2g00660 (TcsB)	-	AO090011000093	ATEG 07365	NFIA 032940
	Mak1*	AN3101.3	ACLA 040370	AFL2G 00704	Afu3g12530	e gw1 2.30	AO090005000715	ATEG 04140	NFIA 064770
	Mak2*	AN3102.3	ACLA_040380	AFL2G_00703	Afu3g12550	fge1_pg_C_2000617	AO090005000714	ATEG_04139	NFIA_064760
		-	-	-	-	-	-	-	NFIA_072160
	Mak2*	AN5296.3 (TcsA)	ACLA_083970	AFL2G_04130	Afu6g10240 (Fos-1)	e_gw1_6.721	AO090023000259	ATEG_02495	NFIA_055960
		-	-	-	-	e_gw1_4.144	-	-	-
	Mak2*	AN4479.3 (NikA)	ACLA 090930	AFL2G 08246	Afu2g03560 (Bos1)	fge1 pg C 5000177	AO090120000228	ATEG 09534	NFIA 035930
Histidine-containing phosphotransfer intermediate	Ypd1p	AN2005 (YpdA)	ACLA_049490	AFL2G_01854	Afu4g10280	An04g06570	AO090003001194	ATEG_00699	NFIA_105780
Response regulator	Ssk1p	AN7697.3 (SskA)	ACLA_012140	AFL2G_06337	Afu5g08390	An03g04670	AO090701000762	ATEG_08259	NFIA_078890
		-	-	AFL2G_12585	-	-	-	-	-
	Prr1*	AN3688.3 (SrrA)	ACLA_085770	AFL2G_02624	Afu6g12520 (afSKN7)	fge1_pg_C_3000205	AO090003000363	ATEG_03268	NFIA_058480

MAPKKK	**Ssk2p**	**AN10153.3 (SskB)**	**ACLA_024570**	**AFL2G_07670**	**Afu1g10940**	**fge1_pg_C_3000791**	**AO090038000312**	**ATEG_00291**	**NFIA_014690**
MAPKK	**Pbs2p**	**AN0931.3 (PbsB)**	**ACLA_019390**	**AFL2G_01045**	**Afu1g15950**	**est_fge1_pg_C_10308**	**AO090005001093**	**ATEG_05187**	**NFIA_009350**
MAPK	**Hog1p**	**AN1017.3 (HogA/SakA)**	**ACLA_011450**	**AFL2G_06243**	**Afu1g12940 (SakA)**	**e_gw1_10.301**	**AO090020000466**	**ATEG_00489**	**NFIA_012610**
	MpkC**	**AN4668.3 (MpkC)**	**ACLA_022520**	**AFL2G_10858**	**Afu5g09100 (MpkC)**	**fge1_pg_C_5000454**	**AO090701000642**	**ATEG_06557**	**NFIA_078210**
Regulation of the HOG pathway	**Ptp2p, Ptp3p**	**AN6982.3 (PtpA)**	**ACLA_053640**	**AFL2G_12456**	**Afu4g04710**	**e_gw1_8.145**	**AO090206000016**	**ATEG_02763**	**NFIA_028630**
	Pyp2*	**AN4896.3**	**ACLA_038900**	**AFL2G_02356**	**Afu3g10970**	**fge1_pm_C_1000613**	**AO090003000651**	**ATEG_04624**	**NFIA_066280**
	Ptc1p	**AN6892.3**	**ACLA_016330**	**AFL2G_08466**	**Afu5g13340**	**est_fge1_pg_C_80382**	**AO090120000479**	**ATEG_06173**	**NFIA_073700**
	Ptc2p, Ptc3p	**AN1358.3**	**ACLA_026190**	**AFL2G_01488**	**Afu1g09280**	**gw1_3.574**	**AO090005001595**	**ATEG_08554**	**NFIA_016350**
Nuclear importin or exportin	**Gsp1p, Gsp2p**	**AN5482.3 (RanA)**	**ACLA_086550**	**AFL2G_02536**	**Afu6g13300**	**An08g10060**	**AO090003000454**	**ATEG_03365**	**NFIA_059220**
	Nmd5p	**AN6006.3**	**ACLA_069050**	**AFL2G_05386**	**Afu2g10010**	**An16g05050**	**AO090011000635**	**ATEG_04435**	**NFIA_085430**
	Crm1p	**AN1401.3 (KapK)**	**ACLA_026800**	**AFL2G_01536**	**Afu1g08790**	**est_GWPlus_C_32422**	**AO090005001650**	**ATEG_08495**	**NFIA_016940**
HogA/SakA targets[c]	**Rck2p**	**AN4483.3**	**ACLA_091030**	**AFL2G_08251**	**Afu2g03490**	**An07g07970**	**AO090120000235**	**ATEG_09539**	**NFIA_035840**
	Tup1p	**AN6505.3 (RcoA)**	**ACLA_095390**	**AFL2G_05678**	**Afu6g05150**	**An15g00140**	**AO090701000021**	**ATEG_05660**	**NFIA_051720**
	Cyc8p	**-**	**ACLA_070780**	**AFL2G_06816**	**Afu2g11840**	**est_fge1_pm_C_20065**	**AO090026000459**	**ATEG_01125**	**NFIA_087070**
	Atf1*	**AN2911.3 (AtfA)**	**ACLA_039220**	**AFL2G_02323**	**Afu3g11330**	**An02g07070**	**AO090003000685**	**ATEG_04664**	**NFIA_065970**
	Msn2p, Msn4p	**AN1652.3 (MsnA)**	**ACLA_048440**	**AFL2G_04479**	**Afu4g09080**	**An04g03980**	**AO090023000650**	**ATEG_05308**	**NFIA_107090**
	Sgd1p	**AN4581.3**	**ACLA_092840**	**AFL2G_05233**	**Afu2g01980**	**e_gw1_5.86**	**AO090011000468**	**ATEG_05716**	**NFIA_034350**
	Rpd3p	**AN4493.3 (RpdA)**	**ACLA_091130**	**AFL2G_08263**	**Afu2g03390**	**An07g07850**	**AO090012000132**	**ATEG_09554**	**NFIA_035740**
		-	**-**	**AFL2G_03062**	**-**	**-**	**-**	**-**	**-**

AtfA targets[c,d]	Cta3*	AN10982.3	ACLA_094630	AFL2G_06033	Afu2g01320	fge1_pm_C_11000239	AO090701000406	ATEG_08161	NFIA_033620
		AN1628.3	ACLA_096890	AFL2G_04426	Afu4g09440	fge1_pm_C_6000111	AO090023000590	ATEG_07100	NFIA_106580
		AN6642.3	-	AFL2G_10132	Afu6g03690	-	AO090009000591	ATEG_05265	NFIA_050200
	Atf1*	AN2911.3 (AtfA)	ACLA_039220	AFL2G_02323	Afu3g11330	An02g07070	AO090003000685	ATEG_04664	NFIA_065970
	Pyp2*	AN4896.3	ACLA_038900	AFL2G_02356	Afu3g10970	fge1_pm_C_1000613	AO090003000651	ATEG_04624	NFIA_066280
	Gpd1*	AN0351.3 (GfdA)	ACLA_032280	AFL2G_00859	Afu1g02150	e_gw1_1.1679	AO090005000883	ATEG_04878	NFIA_022500
	Gpd2*	AN6792.3 (GfdB)	ACLA_080090	AFL2G_05589	Afu2g08250	fge1_pm_C_6000321	AO090011000879	ATEG_02234	NFIA_083910
	Ctt1*	AN9339.3 (CatB)	ACLA_062020	AFL2G_08106	Afu3g02270	est_GWPlus_C_12396	AO090120000068	ATEG_07477	NFIA_003430
	Gpx1*	AN2846.3	ACLA_040140	AFL2G_00727	Afu3g12270	An02g08110	AO090005000739	ATEG_04161	NFIA_065020
		-	-	AFL2G_12085	-	-	-	-	-
SsrA targets[c,d]	Trx2p	AN0170.3 (ThiO/TrxA)[e]	ACLA_014400	AFL2G_06605	Afu5g11320	An01g02500	AO090026000708	ATEG_07726	NFIA_075950
	Trr1p	AN3581.3 (TrxB/TrxR)	ACLA_051990	AFL2G_10383	Afu4g12990	An01g08570	AO090009000289	ATEG_03181	NFIA_103360
	Cta3*	AN10982.3	ACLA_094630	AFL2G_06033	Afu2g01320	fge1_pm_C_11000239	AO090701000406	ATEG_08161	NFIA_033620
		AN1628.3	ACLA_096890	AFL2G_04426	Afu4g09440	fge1_pm_C_6000111	AO090023000590	ATEG_07100	NFIA_106580
		AN6642.3	-	AFL2G_10132	Afu6g03690	-	AO090009000591	ATEG_05265	NFIA_050200
	Och1p	AN4716.3	ACLA_011950	AFL2G_08225	Afu5g08580	e_gw1_5.365	AO090120000208	ATEG_05779	NFIA_078700
	Ctt1p	AN9339.3 (CatB)[f]	ACLA_062020	AFL2G_08106	Afu3g02270	est_GWPlus_C_12396	AO090120000068	ATEG_07477	NFIA_003430
	Gpx1p, Gpx2p	AN2846.3	ACLA_040140	AFL2G_00727	Afu3g12270	An02g08110	AO090005000739	ATEG_04161	NFIA_065020
		-	-	AFL2G_12085	-	-	-	-	-
Sgd1p target[c,d]	Gpd1p	AN0351.3 (GfdA)	ACLA_032280	AFL2G_00859	Afu1g02150	e_gw1_1.1679	AO090005000883	ATEG_04878	NFIA_022500
Smp1p target[c,d]	Stl1p	AN9168.3	ACLA_064920	AFL2G_10659	Afu8g05710	An14g02740	AO090020000696	ATEG_01874	NFIA_098490

MsnA targets[c,d]	**Gpd1p**	**AN0351.3 (GfdA)**	**ACLA_032280**	**AFL2G_00859**	**Afu1g02150**	**e_gw1_1.1679**	**AO090005000883**	**ATEG_04878**	**NFIA_022500**
	Ald2p	**AN9034.3**	**ACLA_043350**	**AFL2G_06572**	**Afu8g02310**	**-**	**AO090026000741**	**ATEG_08300**	**NFIA_095930**
	Gpp2p	**AN1216.3 (GppA)**	**ACLA_024960**	**AFL2G_04374**	**Afu1g10570**	**fge1_pg_C_3000140**	**AO090038000367**	**ATEG_00249**	**NFIA_015080**
	Ctt1p	**AN9339.3 (CatB)**	**ACLA_062020**	**AFL2G_08106**	**Afu3g02270**	**est_GWPlus_C_12396**	**AO090120000068**	**ATEG_07477**	**NFIA_003430**
RcoA targets[c,d]	**Gpd1p**	**AN0351.3 (GfdA)**	**ACLA_032280**	**AFL2G_00859**	**Afu1g02150**	**e_gw1_1.1679**	**AO090005000883**	**ATEG_04878**	**NFIA_022500**
	Ald2p	**AN9034.3**	**ACLA_043350**	**AFL2G_06572**	**Afu8g02310**	**-**	**AO090026000741**	**ATEG_08300**	**NFIA_095930**
	Ctt1p	**AN9339.3 (CatB)**	**ACLA_062020**	**AFL2G_08106**	**Afu3g02270**	**est_GWPlus_C_12396**	**AO090120000068**	**ATEG_07477**	**NFIA_003430**
		AN10982.3	**ACLA_094630**	**AFL2G_06033**	**Afu2g01320**	**fge1_pm_C_11000239**	**AO090701000406**	**ATEG_08161**	**NFIA_033620**
	Cta3*	**AN1628.3**	**ACLA_096890**	**AFL2G_04426**	**Afu4g09440**	**fge1_pm_C_6000111**	**AO090023000590**	**ATEG_07100**	**NFIA_106580**
		AN6642.3	**-**	**AFL2G_10132**	**Afu6g03690**	**-**	**AO090009000591**	**ATEG_05265**	**NFIA_050200**

[a] - Unless otherwise indicated, *S. cerevisiae* stress-response proteins are shown. Please note that the homology between the *A. nidulans* ShoA, YpdA, PtpA, MsnA, AtfA, ThiO/TrxA and GppA and the corresponding *S. cerevisiae* or *S. pombe* genes were relatively low, characterized with *E*-values higher than 1E-40 (Table 3). Superscripts [*] and [**] stand for *S. pombe* and *A. nidulans* stress-response proteins, respectively.
[b] - The Sho1p-branch of osmotic stress sensing is not functional in *A. nidulans*.
[c] - Hypothetical regulations based on yeast stress models.
[d] - Target proteins are regulated at the level of transcription.
[e] - Homologies between *A. nidulans* ThiO/TrxA and *A. clavatus* (7E-32), *A. flavus* (6E-39), *A. fumigatus* (3E-34), *A. niger* (3E-40), *A. oryzae* (1E-36), *A. terreus* (3E-45) and *N. fischeri* (7E-34) orthologous thioredoxins are shown in the parentheses. According to the $E \leq$ 1E-40 cut-off rule, only thioredoxins of *A. nidulans*, *A. niger* and *A. terreus* are shown in the Supplementary Tables.
[f] - Verified target (Hagiwara et al., 2007; Vargas-Pérez et al., 2007).

Table 3
Putative *A. nidulans* orthologues with relatively low (*E* > 1E-40) homology to yeast osmotic and oxidative stress-response proteins

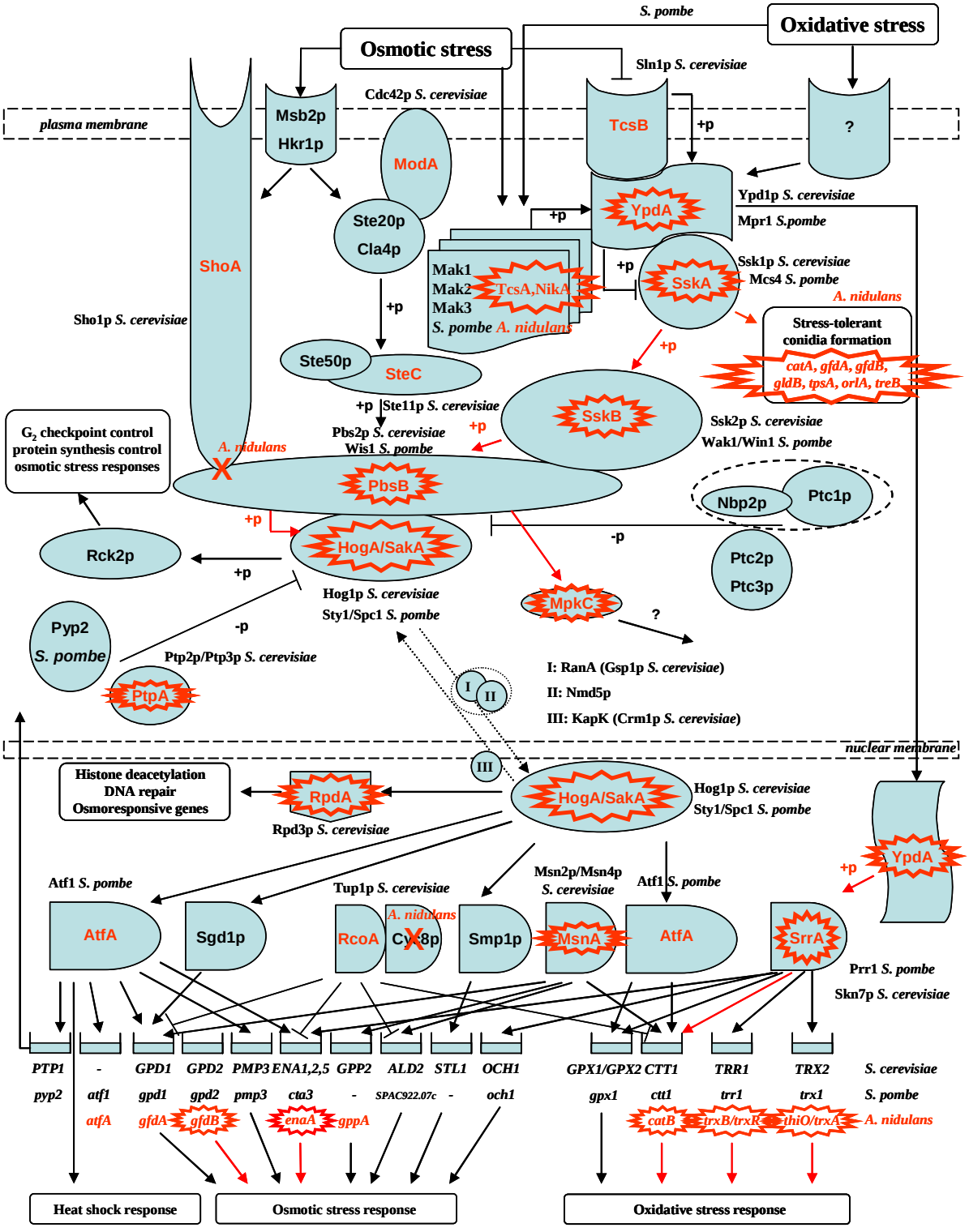
Physiological function	Yeast stress-response proteins	Putative *A. nidulans* orthologues	*E*-value[b]
Sho1p-like stress sensing and signalling branch[c]	Sho1p	AN7698.3 (ShoA)	9E-37
	Msb2p	AN7041.3	1E-18
	Ste50p	AN7252.3	6E-17
Histidine-containing phosphotransfer intermediate	Ypd1p	AN2005.3 (YpdA)[d]	5E-18
Regulation of the HOG pathway	Ptp2p	AN6982.3 (PtpA)[e]	3E-32
	Nbp2p	AN3819.3	5E-17
SakA/HogA targets[f]	Msn2p	AN1652.3 (MsnA)	1E-16
	Msn4p	AN1652.3 (MsnA)	2E-17
	Smp1p	AN2984.3	1E-25
AtfA targets[f,g]	Atf1*	AN2911.3 (AtfA)	1E-36
	Pmp3*	AN2312.3	9E-13
SrrA target[f,g]	Trx2p	AN0170.3 (ThiO/TrxA)[d]	2E-26
MsnA target[f,g]	Gpp2p	AN1216.3 (GppA)	1E-37
Other stress-response transcription factors	Yap1p	AN7513.3	5E-13
	Sko1p[h]	AN2911.3 (AtfA)	1E-09
	Hot1p	-	-
	Msn1p	-	-

[a] - Unless otherwise indicated, *S. cerevisiae* stress-response proteins are shown. Superscripts * stand for *S. pombe* stress-response proteins.
[b] - *E*-values are from BLASTP searches performed in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
[c] - The Sho1p-branch of osmotic stress sensing is not functional in *A. nidulans*.
[d] – Functionality is determined (Furukawa et al., 2005; Vargas-Perez et al., 2007; Thön et al., 2007).
[e] - The homology between Ptp3p and PtpA (Table 2) was more significant (*E*-value 4E-42).
[f] - Hypothetical regulations based on yeast analogies.
[g] - Target proteins are regulated at the level of transcription.
[h] - BLASTP search for Sko1p orthologues brought up AtfA bZip proteins in all aspergilli with low homologies (*E*-values 2E-07 – 9E-09).

Legends to the Figures

Figure 1. Osmotic and oxidative stress signal generation, signal transduction, and stress response in *A. nidulans*. Where our knowledge on *Aspergillus* stress response was limited relevant *S. cerevisiae* and/or *S. pombe* stress response data were taken into consideration. The names of *A. nidulans* gene products having been identified are printed in red, meanwhile red-flamed gene products and red arrows indicate experimentally described and verified stress-response-related gene products and interactions. Orthologue of yeast Cyc8p protein and ShoA-PbsA protein-protein interaction, which do not exist in *A. nidulans*, are marked with red Xs. Elements of osmotic stress sensing, signalling and stress response are summarized in Tables 2 and 3.

Figure 1


S. pombe
Oxidative stress
Osmotic stress
Sln1p S. cerevisiae
Cdc42p S. cerevisiae
plasma membrane
Msb2p
Hkr1p
ModA
TcsB
?
+p
Ypd1p S. cerevisiae
Mpr1 S.pombe
YpdA
Ste20p
Cla4p
ShoA
Mak1
Mak2
Mak3
TcsA,NikA
S. pombe A. nidulans
SskA
Ssk1p S. cerevisiae
Mcs4 S. pombe
A. nidulans
Sho1p S. cerevisiae
Stress-tolerant conidia formation
catA, gfdA, gfdB, gldB, tpsA, orlA, treB
Ste50p
SteC
Ste11p S. cerevisiae
SskB
Ssk2p S. cerevisiae
Wak1/Win1 S. pombe
Pbs2p S. cerevisiae
Wis1 S. pombe
A. nidulans
PbsB
G2 checkpoint control
protein synthesis control
osmotic stress responses
Nbp2p
Ptc1p
Ptc2p
Ptc3p
Rck2p
HogA/SakA
Hog1p S. cerevisiae
Sty1/Spc1 S. pombe
MpkC
Pyp2
S. pombe
Ptp2p/Ptp3p S. cerevisiae
PtpA
I: RanA (Gsp1p S. cerevisiae)
II: Nmd5p
III: KapK (Crm1p S. cerevisiae)
I
II
III
nuclear membrane
Histone deacetylation
DNA repair
Osmoresponsive genes
RpdA
Rpd3p S. cerevisiae
Atf1 S. pombe
Tup1p S. cerevisiae
Msn2p/Msn4p
S. cerevisiae
AtfA
Sgd1p
RcoA
Cyc8p
Smp1p
MsnA
SrrA
Prr1 S. pombe
Skn7p S. cerevisiae
PTP1
pyp2
atf1
atfA
GPD1
gpd1
gfdA
GPD2
gpd2
gfdB
PMP3
pmp3
ENA1,2,5
cta3
enaA
GPP2
gppA
ALD2
SPAC922.07c
STL1
OCH1
och1
GPX1/GPX2
gpx1
CTT1
ctt1
catB
TRR1
trr1
trxB/trxR
TRX2
trx1
thiO/trxA
S. cerevisiae
S. pombe
A. nidulans
Heat shock response
Osmotic stress response
Oxidative stress response